ORIGINAL RESEARCH

Anti-inflammatory and angiogenic effects of exercise training in cardiac muscle of diabetic mice

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Background: Improved glycemic control and cardiovascular function are major benefits of regular exercise training (ET) in type 2 diabetes. Recent work has demonstrated that ET improves cardiac and vascular functions independent of obesity, inflammation, and glucose control in the diabetic db/db mouse. In this study, we determined whether ET can overcome the effects of elevated inflammatory cytokines and hyperglycemia on markers of cardiac angiogenesis and inflammation in the diabetic mouse.

Methods: Male diabetic db/db mice were assigned to a sedentary and exercise-trained group. Sedentary lean control littermates were used as controls. ET was performed at moderate intensity on a treadmill 5 days a week for a period of 8 weeks. After ET, blood was collected for assay of glucose, hemoglobin (HB and HB_{1AC}), C-reactive protein (CRP), and IL-6. Markers of inflammation and insulin resistance (IL-6, IL-1β, and tumor necrosis factor-alpha [TNF-α]) and angiogenesis (endothelial nitric oxide synthase [eNOS], vascular endothelial growth factor-A [VEGF-A], and hypoxia-inducible factor-1α [HIF-1α]) were measured in hearts.

Results: Diabetic db/db mice remained obese and hyperglycemic after ET. Percent total HB and HB_{1AC} were significantly higher in ET db/db mice compared to sedentary db/db mice, indicating further deterioration of glucose control with ET. Plasma levels of CRP and IL-6 were higher in sedentary db/db mice compared to control mice and were unaffected by ET. However, in the presence of hyperglycemia and elevated plasma cytokines, protein expression of eNOS, mRNA expression of VEGF-A, and HIF-1α was increased in db/db hearts after ET. On the other hand, protein expression of TNF-α and mRNA expression IL-6 and IL-1β was significantly decreased by ET in hearts of db/db mice.

Conclusion: Our results indicate that ET improves cardiac markers of angiogenesis, insulin resistance, and endothelial dysfunction in the db/db mouse. This was observed independently of obesity, hyperglycemia, and the systemic inflammatory state.

Keywords: exercise, db/db, glycated hemoglobin, angiogenesis, inflammation

Introduction

It is well established that a sedentary lifestyle is a major risk factor for metabolic and cardiovascular diseases.[1] Physical inactivity is associated with an increased prevalence of obesity, prediabetes, and type 2 diabetes mellitus (T2DM).[2] Patients with these metabolic disorders are at increased risk of developing coronary artery disease (CAD), stroke, and peripheral artery disease.[3] Decreasing the incidence of these adverse cardiovascular outcomes accompanying these disorders can be accomplished by effective nonpharmacological intervention in the form of regular physical activity. Exercise training (ET) performed on a regular basis decreases the

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risk of CAD and slows the progression of endothelial dysfunction, resulting in improved blood flow and organ perfusion.[4–7]

The benefits of ET on vascular function in diabetes are generally attributed to an improvement in glucose control, plasma lipids, and insulin sensitivity.[4,6,8–10] Vascular function is also improved as a result of angiogenesis, resulting in capillarization and improved blood flow in tissues.[11] In the streptozotocin (STZ) model of T1DM, impaired expression of the angiogenic marker vascular endothelial growth factor A (VEGF) in skeletal muscle is improved with ET.[12–15] Furthermore, ET may improve vascular function by decreasing local expression of inflammatory cytokines,[16] which are known to correlate with insulin resistance and endothelial dysfunction in T2DM.[17–21] Recent studies indicate that chronic ET is beneficial on vascular function in the db/db mouse model of T2DM.[8,22,23] Stimulating angiogenesis and decreasing the expression of cytokines represent an important adaptation to ET as improved blood flow promotes the delivery of insulin for the disposal of glucose. However, the effects of ET on expression of inflammatory cytokines linked to vascular dysfunction and insulin resistance, and markers of angiogenesis in cardiac tissue of db/db mice have yet to be addressed.

In this study, we selected the db/db mouse to investigate the role of ET on the expression of cardiac cytokines and markers of angiogenesis. The db/db mouse is characterized by an obese phenotype and exhibits many of the metabolic aberrations seen in human T2DM, including hyperglycemia and inflammatory activation by plasma and tissue cytokines. Levels of C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), and interleukins are elevated in plasma of db/db mice.[23] Another feature of this model is that obesity, insulin resistance, and inflammatory state are not dramatically improved following ET, whereas benefits on vascular function are reported.[8,22–28] Therefore, in this study, the effects of moderate-intensity ET on tissue inflammatory cytokines linked to vascular dysfunction and insulin resistance and markers for angiogenesis in hearts of db/db mice were determined.

Methods
Mouse model of diabetes
Four-week-old diabetic db/db mice (Jackson Laboratories, Bar Harbor, ME, USA) were used in this study. The db/db mouse is characterized by two mutant copies of the leptin receptor gene (C57BL/KsJ-lept^{db}-lept^{db}) and was selected because of its close resemblance to the human T2DM condition.[29] Many of the metabolic perturbations associated with T2DM are displayed by this model, including a gradual onset, an obese phenotype, hyperglycemia, hyperinsulinemia, systemic inflammation, and left ventricular dysfunction. The lean littermates, which possess one mutant and one normal copy of the leptin (db/+) gene, served as controls. All mice used in this study were cared in accordance with the recommendations in The Guide for the Care and Use of Laboratory Animals, National Institute of Health, Publ. No. 85–23, 1986. The Institutional Animal Care and Use Committee of Midwestern University approved this study.

ET protocol
After a 2-week period of acclimation, db/db mice were randomly assigned to either a sedentary (db/db-sedentary) or an exercise-trained (db/db-exercise) group. ET was performed using an electrically driven treadmill (Exer-3/6 treadmill, Columbus Intr., OH) 5 days per week based on a graded increase in duration and intensity, as previously described.[26] Week 1 consisted of running for 10 mins at 10 m/min, followed by 20 min at 10 m/min for week 2, and 30 mins at 12 m/min for week 3. For weeks 4 to 8, mice rat for 30 mins at 15 m/min. This treadmill belt speed corresponds to an estimated oxygen consumption of ~50 mL/kg/min.[30] Throughout the training period, the incline of the treadmill was kept at 0°. Mice were provided with regular chow (Teklad, Harlan Laboratories, Madison, WI, USA) and water ad libidum and housed under a standard alternating 12-hr light/dark cycle at 22°C.

Blood and tissue sampling
Blood was collected from overnight-fasted mice 48 hrs after the last exercise session to eliminate any potential effects on insulin sensitivity.[31] Between 8 and 11 am, nonanesthetized mice were placed on a warm heating pad for 30 mins to facilitate collection of blood by puncture of the submandibular vein. Blood was collected and centrifuged at 3,000 rpm for 5 mins at 4°C. The plasma was stored at −80°C for assay of glucose using a colorimetric assay kit (Wako Chemicals USA, Richmond, VA, USA) and inflammatory markers (IL-6, CRP) using Elisa techniques according to manufacturer specifications (Alpco; Research and Clinical Immunoassays, Salem, NH, USA). The remaining

erythrocytes were stored at 4°C for the determination of glycated hemoglobin content (Helena Laboratories, Beaumont, TX, USA). Mice were quickly euthanized by cervical dislocation. Hearts were removed and frozen with clamps precooled to the temperature of liquid N_2 for analysis of genes and proteins of interest.

Real-time PCR

RNA for VEGF-A, hypoxia-inducible factor-alpha (HIF-1α), IL-1β, and IL-6 was extracted with Trizol reagent (Invitrogen Life Technologies, Burlington, ON, USA) as reported ealier.[25] Samples were incubated with deoxyribonuclease I (Invitrogen Life Technologies) at 37 ºC for 30 mins to remove genomic DNA. PCR was performed in duplicate or triplicate using the iCycler IQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) and SYBR® green chemistry (Bio-Rad Laboratories). Diluted cDNA was added to a reaction mixture (Green Supermix with 200 nM forward and reverse primers) for amplification. Thermal cycling was set at 95ºC for 2 mins, 40 cycles at 95ºC for 30 s, 60ºC for 30 s, and 72ºC for 30 s. The primers were purchased from Invitrogen Life Technologies. Primer sets served to generate amplicons (Table 1). Following PCR, reaction products were melted (1 min at 95ºC, 55ºC with gradual 1.0ºC increments to 95ºC). Optical data were collected over the duration of the temperature increments. The relative expression of RT-PCR products was determined by the ΔΔCt method.[25] GAPDH was selected as internal control because this gene shows consistent expression relative to other housekeeping genes among the groups in our experiments.

Western blot analysis

Heart samples were homogenized in modified RIPA buffer as previously reported.[25] Samples were centrifuged (10,000 g for 20 mins, 4°C), and protein concentration was measured in the supernatant using a modified Bradford assay. Protein (30 µg) was applied to each well and electrophoresed (2 hr at 130 V; MHC: 20 hr at 140 v) with molecular weight markers (RPN800, Amersham Biosciences, Waltham, MA, USA). The protein bands were transferred onto polyvinylidene fluoride membranes (Hybond-C; Amersham Pharmacia, Piscataway, NJ, USA) at 20 V for 60 mins at room temperature using a transfer buffer. The blots were blocked with blocking buffer (5% nonfat milk in 10 mmol/L Tris pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20). Membranes were probed with specific primary antibodies (endothelial nitric oxide synthase [eNOS], 1:1000, Santa Cruz Biotechnology, Dallas, TX, USA; GLUT4, 1: 10,000, Bio-Rad Laboratories, Inc; TNF-α, 1:2000, Cell Signaling, Beverly, MA, USA). Blots were reprobed with an anti-β-GAPDH antibody (1:20,000; Sigma-Aldrich, St. Louis, MO, USA), washed using tris-buffered saline washing buffer (10 mmol/L Tris pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20), and then incubated with horseradish peroxidase-conjugated immunoglobulin G. Blots were detected by chemiluminescence detection system (RPN2132, Amersham) and visualized by exposure to Kodak X-Omat film. Densitometry measurements were performed using Photoshop 7 software.

Statistical analysis

Statistical analysis was performed using the statistical software package Prism 3.0. ANOVA followed by the Tukey–Kramer post hoc test was applied to analyze differences in groups tested. All values are expressed as mean ± SEM.

Results

Physical characteristics, plasma levels of glucose, and inflammatory cytokines, after 8 weeks of ET, are presented in Table 2. As expected, body weight was significantly higher in diabetic mice compared with lean control mice, confirming the obese state associated with the db/db model. However, ET did not significantly improve the obesity associated with these mice.

Table 1 PCR primer sequences

Gene	Sense primer (5′-3′)	Antisense primer (5′-3′)	Accession no.
VEGF	CACCCACGACAGAAGG	TCACAGTGAACGCTCCC	NM_001025250
HIF-1	AGTCGGACAGCCTCA	TGCTGCCTTGTATGGGA	NM_010431
IL-1β	TCCCCAGCCCTTTTGTTGA	TTAGAACCAAATGTGGCCGTG	NM_008361
IL-6	TCTCCACAAGCGCCTTCG	CTCAGGGCTGAGATGCCG	NM_031168
Actin	ACCAACTGGGACGATATGGAGAAGA	TACGACCAGAGGCATACAGGGACAA	NM_007393
GAPDH	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA	NM_008084

Abbreviations: VEGF, vascular endothelial growth factor; HIF-1α, hypoxia-inducible factor alpha; IL-1β, interleukin 1 beta; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2 The effects of exercise training on selected physical characteristics of db/db mice

Parameter	db/+Control	db/db Sedentary	db/db Exercise
Body weight (g)	25.8±0.5	42.3±1.1*	39.5±1.4*
Heart weight (mg)	114±5	104±2	98±2*
HW/BW (x10³)	4.43±0.21	2.46±0.04*	2.49±0.13*
Plasma glucose (mg/dL)	175±13	478±11*	483±8*
Total GHb (%)	5.6±0.1	10.7±0.2*	11.8±0.4*†
Hb$_{1ac}$ (%)	4.8±0.1	8.3±0.2*	9.0±0.3*†
Plasma CRP (ng/ml)	15.21±0.81	18.22±0.78‡	18.16±1.16
Plasma IL-6 (pg/ml)	1.47±0.70	3.33±0.59	4.37±1.70

Notes: Values are reported as mean ± SEM for 7–9 mice in each group. *$P<0.05$ compared to lean mice; †$P<0.05$ compared to db/db sedentary; ‡$p=0.0501$ compared to lean mice.
Abbreviations: Hb, hemoglobin; HW, heart weight; BW body weight; GHb, glycated hemoglobin. CRP, C-reactive peptide; IL-6, interleukin-6.

Figure 1 The effects of diabetes and exercise training on cardiac eNOS protein expression. Values are reported as mean ± SEM for 4–6 mice per group. *$P<0.05$, compared to lean control mice. †$P<0.05$ compared to db/db sedentary mice.
Abbreviations: eNOS, endothelial nitric oxide synthane; GAPDH, glyceraldehyde-3- phosphate dehydrogenase.

Plasma glucose levels were significantly elevated in diabetic mice compared to lean control mice. Following 8 weeks of ET, db/db mice remained hyperglycemic and total glycated hemoglobin and hemoglobin 1$_{ac}$ levels were significantly higher compared to sedentary diabetic mice. These results suggest that glycemic control further deteriorated as a result of ET.

Plasma levels of IL-6 were higher by ~56% in db/db mice, although this did not reach statistical significance. Plasma IL-6 levels were not affected by ET, but tended to be higher in db/db mice after ET compared to sedentary db/db mice. Plasma CRP levels were significantly higher ($P<0.0501$) in db/db mice compared to control mice and were not affected by ET in db/db mouse.

Expression of eNOS in hearts of db/db hearts following ET is illustrated in Figure 1. A significant reduction in the expression of eNOS was observed in sedentary db/db hearts compared to control hearts. Although ET resulted in a significant increase in the expression of eNOS compared to sedentary db/db mice, expression of this endothelial marker remained lower compared to hearts from lean control mice.

Figure 2 illustrates the effects of ET on mRNA expression of VEGF-A and HIF-1α. Expression of VEGF-A and HIF-α were not altered by the diabetic state. However, increases of nearly 40–45% for both markers of angiogenesis were observed in db/db hearts after ET. However, when comparing the expression of VEGF-A and HIF-1α between lean control and ET-hearts, significant differences were observed.

Expression of TNF-α and GLUT4 was measured to determine whether they are reciprocally related. As illustrated in Figure 3, cardiac TNF-α was significantly
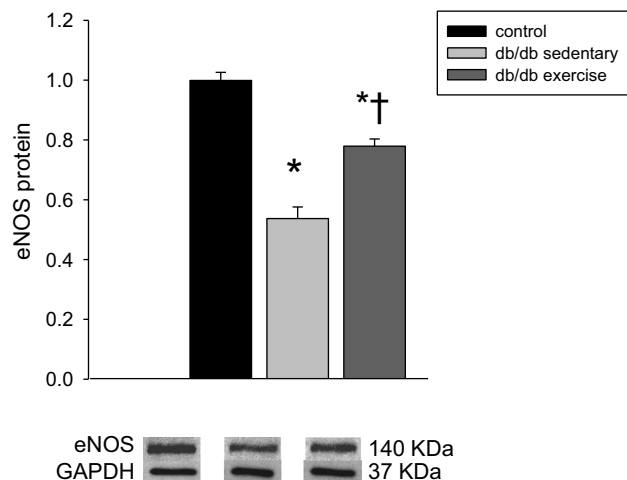
increased in sedentary db/db mice compared with control mice. ET resulted in a significant decrease in the expression of TNF-α. However, decreased expression of TNF-α was not associated with increased GLUT4 content from whole heart homogenates after ET. Gene and protein expressions of GLUT1 were also not affected by the diabetic state or ET (data not provided).

The effects of diabetes and ET on mRNA expression of IL-6 and IL-1β are illustrated in Figure 4. Expression of IL-was was significantly increased in hearts from sedentary db/db mice compared with control mice. A significant reduction in the expression of IL-6 was observed in db/db hearts after ET. There were no differences in IL-1β between control and sedentary db/db hearts. However, IL-1β expression was significantly decreased in db/db hearts after ET.

Discussion

Regular physical activity is a healthy and effective non-pharmacological approach for the management of T2DM. Exercise helps achieve better glucose control, decreases body weight, and improves cardiovascular health. In this study, we used the db/db mouse because of its close similarity to the human condition of T2DM. The onset of diabetes in this model is gradual and is characterized by obesity, insulin resistance, hyperglycemia, systemic inflammation, and cardiac dysfunction. The results of our study indicate that ET for a period of 8 weeks provided cardioprotection in the form of decreased expression of
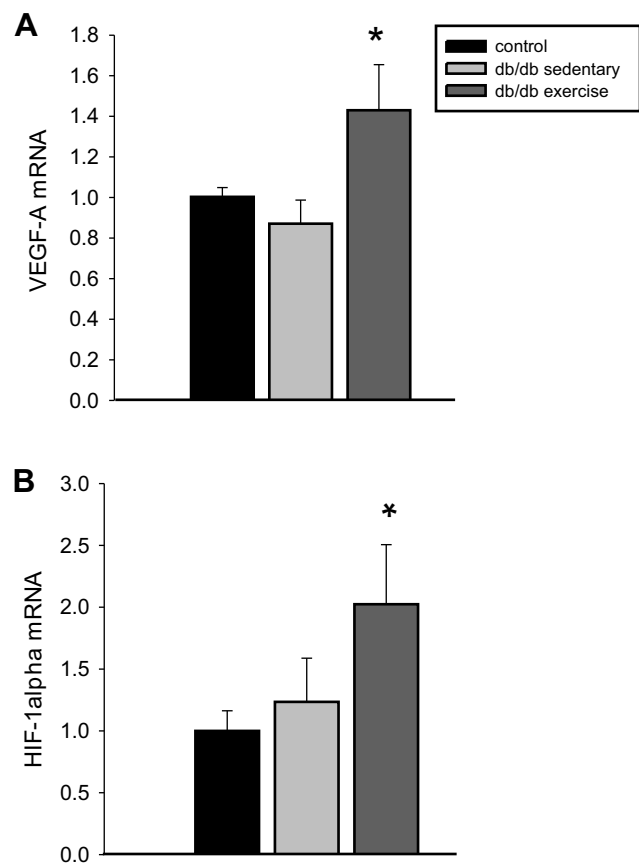
Figure 2 The effects of diabetes and exercise training on cardiac mRNA VEGF-A (panel A) and HIF-1α (panel B) expression. Values are reported as mean ± SEM for 4–6 mice per group. *$P<0.05$ compared to lean control mice.
Abbreviations: VEGF-A, vascular endothelial growth factor; HIF-1α, hypoxia-induced factor.

Figure 3 The effects of diabetes and exercise training on cardiac TNF-α (panel A) and GLUT4 (panel B) protein expression. Values are reported as mean ± SEM for 4–6 mice per group. *$P<0.05$ compared to lean control mice. †$P<0.05$ compared to db/db sedentary mice.
Abbreviations: TNF-α, tumor necrosis factor-alpha; GLUT4, glucose transporter protein 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

genes associated with insulin resistance and endothelial dysfunction and improved gene expression of markers relating to angiogenesis in db/db heart. These benefits afforded by ET occurred independently of glucose control, systemic inflammation, and obesity.

The observation that db/db mice remained obese after ET is consistent with earlier studies.[22,26–28] The reasons for this effect are not known, although it has been suggested that treadmill running may worsen the diabetic state by increasing the levels of stress hormones. By its nature, chronic treadmill running is considered a form of forced exercise known to elicit an exaggerated cortisol response in the db/db mouse,[24–28,32] which is a well-established risk factor contributing to visceral obesity and weight gain in diabetes and obesity,[33,34] On the other hand, low-intensity voluntary running designed to mimic exercise programs for patients with chronic disorders proves to be more efficient in preventing weight gain and decreasing food intake and urinary corticosterone excretion in the db/db mouse.[27,28]
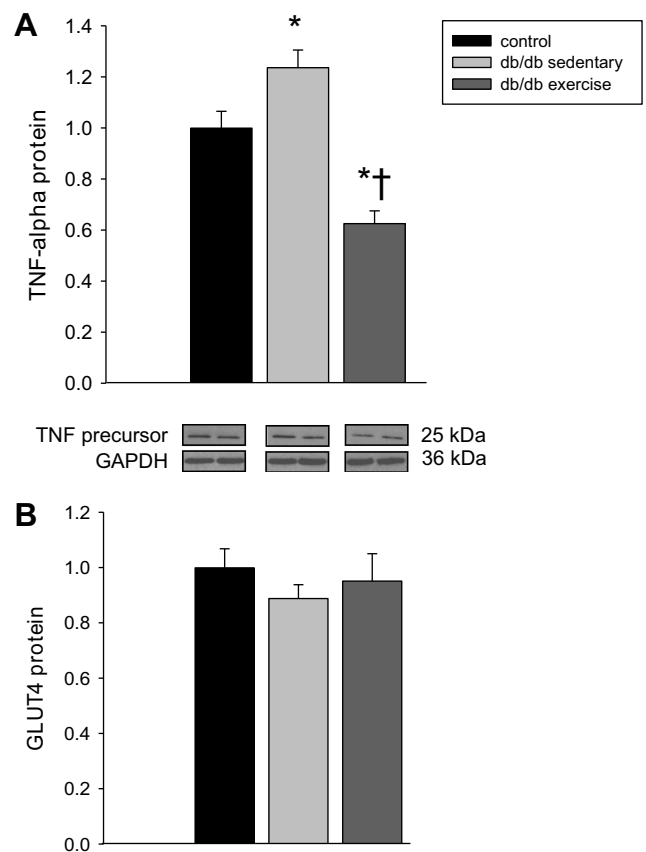
The effects of ET on glucose regulation have been studied in the db/db mouse. Most of these studies showed that the db/db mouse remained either hyperglycemic or demonstrated only slight improvements in blood glucose levels after ET.[8,22–28] Following 8 weeks of ET, db/db mice were hyperglycemic and glycated hemoglobin 1_{ac} levels remained elevated compared to their sedentary diabetic counterparts, indicating that glycemic control further deteriorated from ET. Similar results have been reported after 5 and 12 weeks of treadmill running at the same intensity in the leptin-deficient ob/ob mouse model of severe obesity.[27,28,35] Evidence suggests that these maladaptations to exercise are attributed to an increase in insulin resistance from defective glucose transport and insulin signaling in skeletal muscle.[27] Moreover, the db/db mouse exhibits hypercorticosteronism and increased expression of 11β-hydroxysteroid dehydrogenase type 1,
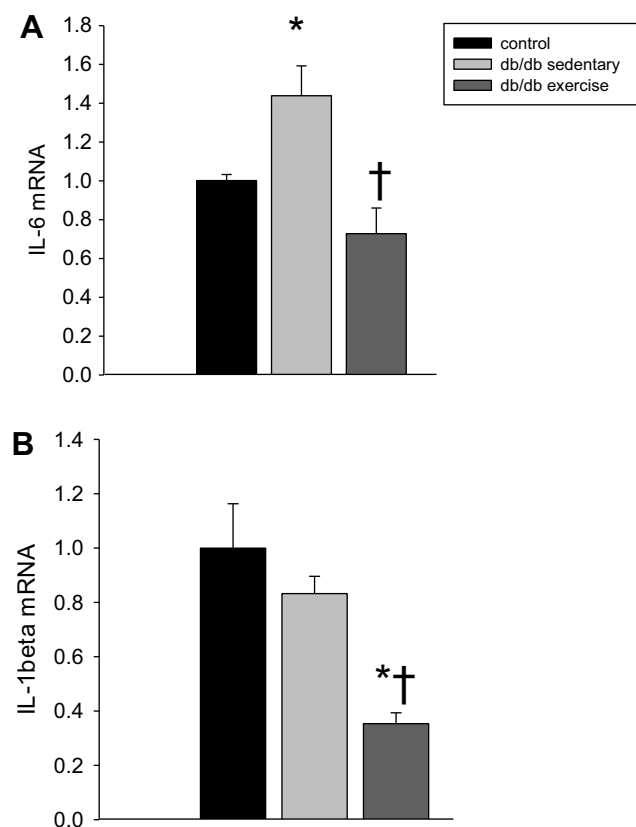
Figure 4 The effects of diabetes and exercise training on cardiac mRNA IL-6 (panel A) and IL-1β (panel B) expression. Values are reported as mean ± SEM for 4–6 mice per group. *$P<0.05$ compared to lean control mice. †$P<0.05$ compared to db/db sedentary mice.

Abbreviation: IL-1β, interleukin-1 beta.

both contributing to elevated rates of hepatic glucose production.[24,32] Acute exercise and chronic ET further increase endogenous glucocorticoid secretion in the db/db mouse, resulting in increased rates of hepatic gluconeogenesis and hyperglycemia.[24,28] This is in sharp contrast to earlier studies in the obese Zucker and Zucker diabetic fatty rat where the hypercorticosteronism and insulin resistance were prevented and an improvement in hepatic glucose production rates and hyperglycemia was observed in response to treadmill running.[36,37] The reasons for these discrepancies are not known, but may relate to differences in the severity of diabetes and adiposity exhibited by the rodents used. Leptin resistance in the db/db mouse may also explain the hyperglycemia since treatment of uncontrolled hypoleptinemic type 1 diabetic rats with leptin normalizes plasma glucose and hepatic glucose production and improves energy expenditure and running activity in obese mice.[38,39]

T2DM is a low-grade inflammatory disease, and patients often have elevated plasma levels of the proinflammatory cytokines CRP and IL-6.[17] CRP is also predictor of diabetes, and levels of CRP in plasma are correlated with insulin resistance, obesity,[40] glycated hemoglobin,[41] and endothelial dysfunction.[42] Further, synthesis of hepatic CRP is mediated by IL-6 and CRP production is induced by hyperglycemia.[43,44] Our results indicate that after moderate ET circulating levels of proinflammatory cytokines remained elevated in db/db mice. These findings are in keeping with recent work in db/db mice where a persistent inflammation was observed after 6 weeks of forced wheel ET, although endothelial function was observed in these mice.[23] It is well established that elevated CRP levels and hyperglycemia inhibit vascular function by decreasing protein expression eNOS.[45–47] Declining levels of eNOS and decreased action of NO are central to the pathogenesis and clinical course of cardiovascular dysfunction. eNOS is also a component of the cardioprotective oxytocin-atrial natriuretic peptide system,[48] which is downregulated in diabetes and linked to cardiomyopathy observed in these mice.[49–51] Our results indicate that the expression of eNOS was significantly increased in diabetic hearts after ET. This is consistent with earlier studies using this mouse model of diabetes and also shows that ET is beneficial on eNOS expression even in the presence of severe hyperglycemia and obesity.[25,26] Although this would suggest improved endothelial function,[52] the functional consequences of this increase in eNOS in response to ET were not determined in this study. However, previous studies have reported improved endothelial function, expressed as vasodilation to acetylcholine in aortae and coronary arteries, in the db/db mouse trained under similar exercise conditions.[8,22,23]

In addition to its role in vascular function, eNOS is a key modulator of angiogenesis in heart.[53,54] Angiogenesis is stimulated as a compensatory response to ischemia and ET in the aging heart.[55] Further, evidence indicates that hyperglycemia inhibits angiogenesis in hearts of diabetic patients.[56] Recent studies demonstrated that ET in the form of wheel or treadmill running increased VEGF-A levels in hearts from STZ-induced type 1 insulinotropic diabetes rats.[12] This effect may explain, in part, the benefits of regular exercise on cardiovascular disease risk and coronary blood in diabetes. Coronary blood is improved with training because of angiogenesis and increased endothelial vasodilation function.[57] Taken together, our results show that the increase in mRNA expression of VEGF-A expression is

consistent with the changes in eNOS and occur independently of hyperglycemia. In addition to the metabolic abnormalities in substrate utilization, inflammation, and disturbances in cardiac function, hearts from diabetic models also display impaired responses to hypoxia.[58] Hypoxia protects the myocardium from lethal hypoxic and ischemic conditions by activating HIF-1α, a key transcription factor involved in the expression of VEGF.[59] We measured mRNA expression of HIF-1α to determine whether this was associated with VEGF-A expression observed in db/db hearts after ET. When compared to lean control mice, the increased expression of HIF-1α observed in db/db hearts after ET could explain the training effect on VEGF-A expression.

Increased inflammation in T2DM is known to contribute to disturbances in endothelial function, glucose metabolism, and cardiac function.[17–61] TNF-α and IL-6 levels are increased in the endothelial vasculature of hearts of diabetic mice and have been identified as key mediators precipitating vascular dysfunction. Expression of TNF-α and IL-6 was increased in hearts of sedentary db/db mice and ET significantly decreased expression of these cytokines. Decreased TNF-α expression has been previously reported in db/db mice after 10 weeks of ET, and this was associated with improved vasodilatory function of coronary arteries and a 20–25% decrease in serum IL-6,[61] suggesting that ET is protective and inhibits the progression of vascular dysfunction.[62] Moreover, studies indicate that TNF-α also regulates peripheral glucose metabolism. Evidence for this role is supported in mice lacking TNF-α function, demonstrating improved insulin sensitivity following high-fat feeding, and similarly, in which an intravenous infusion of TNF-α inhibits insulin-mediated glucose uptake in healthy subjects.[63,64] However, in cardiac tissue of exercise-trained db/db mice, decreased TNF-α content was not accompanied with reciprocal changes in the expression of either GLUT4 protein content or GLUT1 gene and protein expression of (data not provided). However, one might expect that the reduction in these cytokines after ET can potentially lead to an improvement in vascular function and glucose metabolism, and that the changes in gene and protein expression induced from ET may appear to counter the diabetic state. Admittedly, as a limitation of this study, the effects of ET on these proinflammatory cytokines on vascular function and peripheral glucose metabolism in this study must be considered.

Conclusion

In this study, we show that 8 weeks of moderate-intensity ET does not improve glucose control and plasma levels of inflammatory cytokines, but is associated with increased expression of key angiogenic markers, possibly by enhancing NO availability in hearts of diabetic mice. On the other hand, expression of key cytokines involved in the progression of endothelial dysfunction and insulin resistance in cardiac muscle was reduced in response to ET. The observation that these beneficial effects of ET occurred independent of obesity, hyperglycemia, and the inflammatory state suggests that ET is protective in nature.

Acknowledgments

Disclosure

The authors report no conflict of interest in the work.

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